Chapter 1

An Introduction to Natural Products Isolation

Satyajit D. Sarker and Lutfun Nahar

Abstract

Natural products, well known for unique chemical diversity and bioactivity, have continued to offer templates for the development of novel scaffolds of drugs. With the remarkable developments in the areas of separation science, spectroscopic techniques, microplate-based ultrasensitive in vitro assays and high-throughput screening (HTS) technologies, natural products research has gained momentum in recent years. The pre-isolation analyses of crude extracts or fraction from different natural matrices, isolation, online detection and dereplication of natural products, studies on chemotaxonomy and biosynthesis, chemical finger-printing, quality control of herbal products, and metabolomic studies have now become much easier than ever before because of the availability of a number of modern sophisticated hyphenated techniques, e.g., GC–MS, LC–PDA, LC–MS, LC–FTIR, LC–NMR, LC–NMR–MS, and CE–MS. This introductory chapter presents a general overview of the processes involved in natural products research, starting from extraction and isolation to elucidation of the structures of purified natural products and their bioactivity.

Key words: Natural products, Secondary metabolite, Extraction, Isolation, Structure determination, Bioassay

1. Introduction

Simply, products of natural origins can be termed as "natural products." Natural products can be

(a) an entire organism, e.g., a plant, an animal, or a microorganism, that has not been gone through any processing or treatment other than simple process of preservation, e.g., drying;

(b) part of an organism, e.g., leaves or flowers of a plant, or an isolated animal organ;

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(c) an extract of an organism or part of an organism, and exudates;

(d) pure compounds, e.g., alkaloids, coumarins, flavonoids, glycosides, iridoids, lignans, steroids and terpenoids, isolated from plants, animals, or microorganisms (1).

However, in most cases, the term "natural products" refers to secondary metabolites produced by any living organism; they are small molecules (mol. wt. <2,000 amu), which are apparently not necessary for the survival, growth, development, or reproduction of the organism that produces them. Secondary metabolites are often restricted to a narrow set of species within a phylogenetic group, and include products of overflow metabolism as a result of nutrient limitation, or shunt metabolism produced during idiophase, defense mechanism, or regulator molecules (2). They seem to often play an important role in plant defense against herbivory and other interspecies defense mechanisms. Natural products can be from any terrestrial or marine source: plants [e.g., paclitaxel (Taxol®) from *Taxus brevifolia*], animals (e.g., Vitamin A and D from Cod liver oil) or microorganisms (e.g., penicillin G from *Penicillium notatum*).

1.1. Natural Products: Historical Perspective, Current Status, and Future Prospects

The use of natural products, especially terrestrial higher plants, for healing is as ancient and universal as medicine itself. Several well-known plant species, e.g., licorice (*Glycyrrhiza glabra*), myth (*Commiphora* species), and poppy capsule latex (*Papaver somniferum*), were mentioned as medicinal herbs in the first known written record on clay tablets from Mesopotamia in 2600 BC (3). The therapeutic use of plants can easily be traced back to the Sumerian civilization, and 400 years before the Common Era, it is recorded that Hippocrates utilized approximately 400 different plant species for medicinal purposes (2). Natural products played a prominent role in ancient traditional medicine systems, like the Chinese, the Ayurvedic (see Note 1) and the Egyptian traditional medicines, and are still in common use today for the treatment of various diseases. According to the World Health Organization (WHO), over 75% of people still rely on plant-based traditional medicines for primary health care in underdeveloped or developing countries (2). Table 1 presents a brief summary of history of natural products medicine.

Mother Nature has been a major source of therapeutic agents for thousands of years, and an impressive number of modern drugs have been derived from natural sources, many based on their use in traditional medicine. Historical experiences with plants as therapeutic agents have helped to discover single chemical entities with therapeutic value in modern medicine (4). Plants, especially those with ethnomedicinal uses, have been the primary sources of medicines for early drug discovery. It has been reported that uses

Table 1
History of natural products medicine

Period	Type	Description
>3000 BC	Ayurveda Chinese Traditional Medicine	Introduced medicinal properties of plants and other natural products
1550 BC	*Ebers Papyrus*	Presents a large number of crude drugs from natural sources (e.g., castor seeds and gum Arabic)
460–377 BC	Hippocrates "The Father of Medicine"	Described several plants and animals that can be the sources of medicine
370–287 BC	Theophrastus	Described several plants and animals that can be the sources of medicine
23–79 AD	Pliny the Elder	Described several plants and animals that can be the sources of medicine
60–80 AD	Dioscorides	Wrote, "De Materia Medica" which described more than 600 medicinal plants
131–200 AD	Galen	Practised botanical medicines (Galenicals) and made them popular in the west
Fifteenth century	Kräuterbuch (herbals)	Presented information and pictures of medicinal plants

of 80% of 122 plant-derived drugs were related to their original ethnomedicinal purposes (4). Before the advent of high-throughput screening (HTS) and the post-genomic era, more than 80% of drug substances were natural products or inspired by a natural products (5).

Over the last century, a number of top selling drugs have been developed from natural products; vincristine from *Vinca rosea*, morphine from *P. somniferum*, and Taxol® from *T. brevifolia* are just to name a few. About 40% of all modern drugs that are in use today have been developed from natural products. According to Cragg et al. (6), 39% of all 520 new approved drugs in 1983–1994 were natural products or derived from natural products, and 60–80% of antibacterial and anticancer drugs were from natural origins. In 2000, approximately 60% of all drugs in clinical trials for the multiplicity of cancers were natural products. In 2001, eight (simvastatin, pravastatin, amoxycillin, clavulanic acid, clarithromycin, azithromycin, ceftriaxone, cyclosporin, and paclitaxel) of the 30-top selling medicines were natural products or derived from natural products and these eight drugs together totaled US $16 billion in sales. Almost 50% of the drugs approved since 1994 are based on natural products (5). Between 2001 and 2005, 23

new drugs derived from natural products were introduced for the treatment of bacterial and fungal infections, cancer, diabetes, dyslipidemia, atopic dermatitis, Alzheimer's disease, and genetic diseases, such as tyrosinaemia and Gaucher disease (7). At least 13 natural product-derived drugs were approved between 2005 and 2007, and five of those, exenatide, ziconotide, ixabepilone, retapamulin, and trabectedin, represented the first members of novel classes of drugs (5).

In addition to natural product-derived modern medicine, natural products are also used directly in the "natural" pharmaceutical industry that has been growing rapidly in Europe and North America, as well as in traditional medicine programs being incorporated into the primary health care systems of Mexico, The People's Republic of China, Nigeria, and other developing countries (2). The popularity of herbal medicines in the form of food supplements, nutraceuticals, complementary and alternative medicine, has risen sharply in recent years.

The value of natural products in new drug discovery will continue to be significant in the years to come, mainly because of their inherent unmatched chemical structural diversity, "drug-like" properties (see Note 2) and proven credentials with regard to

(a) the rate of introduction of new chemical entities of wide structural diversity, including serving as templates for semisynthetic and total synthetic modification;

(b) the number of diseases treated or prevented by these substances;

(c) their frequency of use in the treatment of disease (4).

It is envisaged that natural products will continue to contribute to the search for new drugs in three different ways, by

(a) acting as new drugs that can be used in an unmodified state, e.g., vincristine from *Catharanthus roseus*;

(b) providing chemical "building blocks" or "scaffolds" used to synthesize more complex molecules, e.g., diosgenin from *Dioscorea floribunda* for the synthesis of oral contraceptives;

(c) indicating new modes of pharmacological action that allow complete synthesis of novel analog, e.g., synthetic analogs of penicillin from *P. notatum* (2).

Only a small fraction of the world's biodiversity has been explored for drug discovery to date. There are at least 250,000 species of higher plants that exist on this planet, but merely a 5–10% of these terrestrial plants have ever been investigated. In addition, reinvestigation of previously investigated plants has continued to produce new bioactive compounds that have drug potential. Much less is known about marine organisms than other sources of natural products. Natural product resources, especially from the marine environment, are largely unexplored. However, research to date

has established that marine organisms could be a valuable source for novel bioactive compounds for drug discovery and development. The discovery of a number of highly cytotoxic compounds, e.g., cephalostatins, crellastatins, and ritterazines from marine sponges has opened up the possibilities of discovering new anticancer drugs from marine organisms (8).

With the development of new molecular targets, there is an increasing demand for novel molecular diversity for screening. Natural products will certainly play a crucial role in meeting this demand through the continued investigation of world's biodiversity, much of which remains unexplored (9). With less than 1% of the microbial world currently known, advances in technologies for microbial cultivation and the extraction of nucleic acids from environmental samples from soil and marine habitats, will offer access to an untapped reservoir of genetic and metabolic diversity (10). This is also true for nucleic acids isolated from symbiotic and endophytic microbes associated with terrestrial and marine macroorganisms.

Advent, introduction, and development of several new, highly specific and ultrasensitive in vitro bioassay techniques, chromatographic methods, and spectroscopic techniques, especially NMR, have made it much easier to screen, isolate, and identify potential drug "lead" compounds quickly and precisely. Automation of these methods now makes natural products viable for HTS (2).

1.2. Strategies in Natural Products Research

Strategies for research in the area of natural products have evolved dramatically over the last few decades in order to keep up with the pace of developments and changes in other related areas. However, classical and more traditional approaches to natural products research are still valid and used routinely. The strategies of natural products research may be divided broadly into two categories as outlined below (2).

1.2.1. Older Strategies

(a) Predominantly focused on chemistry of compounds from natural sources, but not on activity.

(b) Straightforward isolation and identification of compounds from natural sources, mainly from higher plants, followed by biological activity testing (mainly in vivo).

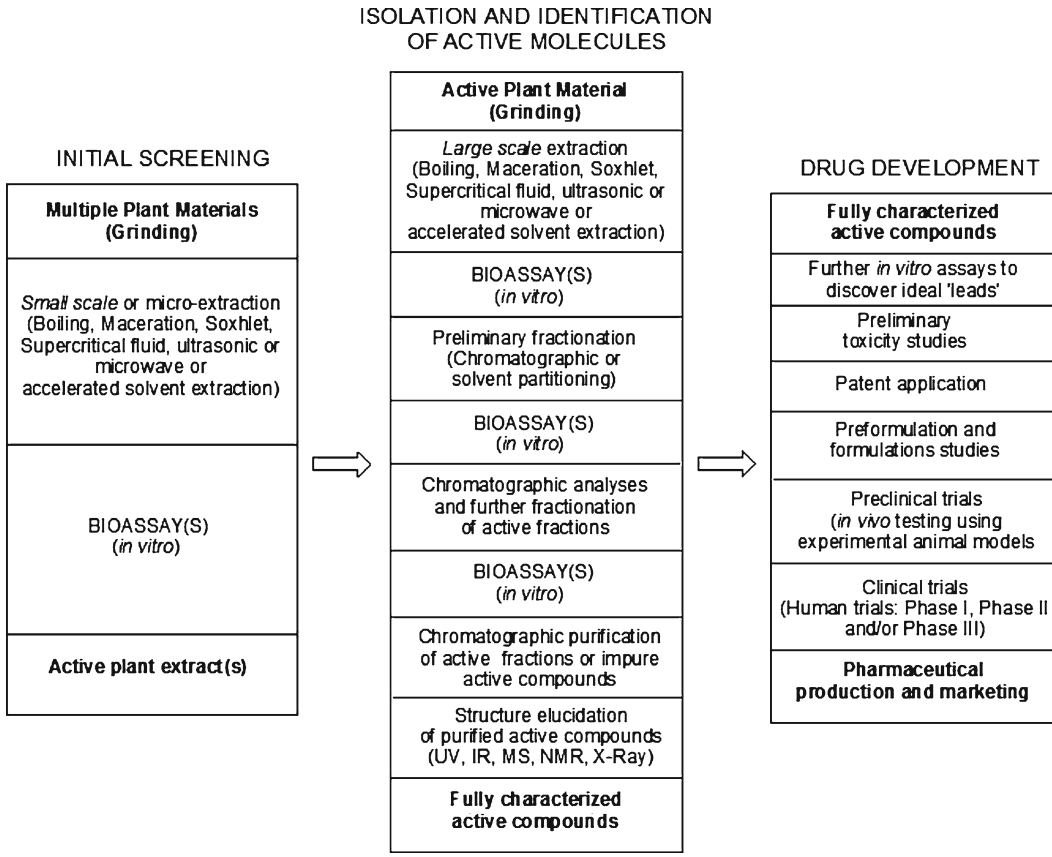
(c) Chemotaxonomic investigation.

(d) Selection of organisms primarily based on ethnopharmacological information, folklore, or traditional uses.

1.2.2. Modern Strategies

(a) Bioassay-guided (mainly in vitro) isolation and identification of active "lead" compounds from natural sources (Scheme 1).

(b) Production of dereplicated "natural products libraries" for HTS screening.



Scheme 1. A generic protocol for drug discovery and development from plants using a bioassay-guided approach.

(c) Production of active compounds in cell or tissue culture, genetic manipulation, and natural combinatorial chemistry.

(d) More focused on bioactivity.

(e) Introduction of the concept of "chemical fingerprinting" and "metabolomics."

(f) Selection of organisms based on ethnopharmacological information, folklore, or traditional uses, and also randomly selected.

(g) Utilization of other natural sources other than higher plants, particularly marine organisms.

1.3. Final Words

There are several well-established methods for extraction and isolation of natural products from various sources available nowadays. An appropriate protocol for extraction and isolation can only be designed once the target compound(s) or the overall aim has been established. It is always helpful to obtain information, as much as possible, on the chemical and physical nature of the compound(s)

to be isolated. For unknown natural products, sometimes it may be necessary to try out pilot extraction and isolation methods to find out the best possible method. At the time of choosing a method, one should be open-minded enough to appreciate and weigh up the advantages and disadvantages of all available methods, particularly focusing on their efficiency and obviously the total cost involved. Continuous progress in the area of separation science has increased the variety and variability of the extraction and isolation methods that can be utilized effectively in the extraction and isolation of natural products. For any natural products researcher, it is therefore crucial to become well-versed with the newer approaches. In most cases, extraction and isolation of natural product is followed by structure elucidation or confirmation of the identity of purified components. With the introduction of and advances in various hyphenated techniques (see Chapter 12), it is now possible to determine the structure of the compound as a separation is performed, without isolation and purification (2). Over the last few decades, with the phenomenal progress in the area of mass spectrometry and NMR, it has now become possible to deduce the structure of a compound in microgram amounts (2), and thus added to the blurring of the boundaries between analytical and preparative methods.

2. Materials

Suitable solvents, e.g., *n*-hexane, liquid carbon-di-oxide (CO_2), dichloromethane (DCM), *n*-butanol, ethanol (EtOH), methanol (MeOH) or water, and an appropriate extraction apparatus, e.g., Soxhlet, are required for extraction.

For fractionation of a crude extract, appropriate solvents, e.g., *n*-hexane, petroleum ether, chloroform, ethyl acetate (EtOAc), and/or *n*-butanol for solvent partitioning, and suitable chromatographic systems, e.g., vacuum liquid chromatography (VLC), flash chromatography (FC), column chromatography (CC), size exclusion chromatography (SEC), solid-phase extraction (SPE), droplet counter-current chromatography (DCC) or preparative high performance chromatography (prep-HPLC) set up together with suitable mobile phase (solvents) and stationary phase, e.g., silica gel, C_{18} silica are necessary.

Similarly, chromatographic systems, e.g., thin layer chromatography (TLC), preparative thin layer chromatography (PTLC), CC, DCC, semipreparative or preparative high performance chromatography (semiprep or prep-HPLC), and suitable mobile phase (solvents) and stationary phase (see Chapters 6–15) are also required for natural products isolation.

For structure elucidation, ultraviolet–visible spectrophotometer (UV–vis), infrared spectrophotometer (IR), mass spectrometer (MS),

and/or nuclear magnetic resonance spectrometer (NMR) and corresponding sample preparation tools and solvents are needed.

Bioassay materials are quite variable and depend entirely on the type of bioassay to be performed. For example, the microtiter-based antimicrobial assay using resazurin as an indicator of cell growth (11) requires, mainly 96-well microtiter plates, isosensitest medium or Mueller Hinton medium, microbial strains, resazurin tablets, incubator, centrifuge, normal saline, antibiotic standard (e.g., ciprofloxacin), multichannel micropipette and dimethylsulfoxide (DMSO).

3. Methods

3.1. Extraction

The choice of extraction method depends on the nature of the source material as well as the target compounds (2). Therefore, prior to choosing an extraction method, it is important to decide on the overall target of the extraction. The target of an extraction process may be

(a) an unknown bioactive compound;

(b) a known compound;

(c) a group of structurally related compounds;

(d) all secondary metabolites produced by a particular natural source, which are not produced by a different "control" source, e.g., two species of the same genus or the same species grown under different conditions;

(e) identification of all secondary metabolites presents in an organism for chemical fingerprinting or metabolomics study (see Chapter 12).

One should also seek for answers to the questions associated with the expected outcome of the extraction process. Some of those obvious questions are as follows:

(a) Is this extraction for purifying sufficient amount of a compound to characterize it partially or fully? What is the required level of purity (see Note 3)?

(b) Is this to provide enough material for confirmation or denial of a proposed structure of a previously isolated compound (see Note 4)?

(c) Is this to produce as much as possible so that it can be used for further studies, e.g., clinical trial?

A typical extraction process, especially for plant materials (see Chapter 13), incorporates the following steps:

1. Drying and grinding of plant material or homogenizing fresh plant parts (e.g., leaves and flowers) or maceration of total plant parts with a solvent.
2. Choice of solvents:
   (a) Polar extraction: water, EtOH, or MeOH.
   (b) Medium polarity extraction: EtOAc or DCM.
   (c) Nonpolar: *n*-hexane, petroleum ether or chloroform ($CHCl_3$).
3. Choice of extraction method:
   (a) Accelerated solvent.
   (b) Boiling.
   (c) Maceration.
   (d) Microwave.
   (e) Soxhlet.
   (f) Sublimation.
   (g) Supercritical fluid.
   (h) Steam distillation or hydro-distillation.
   (i) Ultrasonic.

Various initial and bulk extraction techniques for natural products are detailed in Chapters 2 and 13 using specific examples.

3.2. Fractionation

A crude extract of a plant, microbe, or animal matrix literally contains a complicated mix of several compounds. A single separation technique is unlikely to produce a pure single compound from the crude extract. Therefore, it is often necessary to initially fractionate the crude extract into various discrete fractions containing a group of compounds of similar polarities or molecular sizes. These fractions may be obvious, physically discrete divisions, such as the two phases of a liquid–liquid extraction, or they may be the contiguous eluate from a chromatography column, e.g., CC, circular centrifugal chromatography, DCC, FC, prep-HPLC, SEC, SPE, or VLC (see Chapters 6, 7, 9, 10, and 13–15). However, for initial fractionation of any crude extract, one must not generate too many fractions because it may spread the target compound over so many fractions that the fractions containing this compound in low concentrations might evade detection or not show any detectable activity in bioassays in bioassay-guided isolation protocols. It is advisable to collect only a few large, relatively crude fractions and quickly home in on those containing the target compound (2). For finer, and perhaps more meaningful, fractionation of a crude extract, a suitable hyphenated technique, e.g., LC–PDA, can be used (see Chapter 12).

3.3. Isolation

Like extraction, the most important factor to be considered before choosing an isolation protocol is the nature of the target compound(s) present in the crude extracts or fractions. Solubility (hydrophobicity or hydrophylicity), acid–base properties, charge, stability, and molecular size are the key factors of the target molecule(s) that have to be taken into account (2). For the isolation of a known compound, it is not difficult to obtain literature information on its chromatographic behavior, and thus, one can easily choose the most appropriate method for isolation with great degree of confidence. However, it is more difficult to design an isolation protocol for a crude extract where the types of compounds present are unknown or not previously described. In this situation, qualitative chemical tests for the presence of various classes of compounds, e.g., alkaloids, flavonoids, phenolics, or steroids, as well as preliminary TLC (see Chapter 6) or HPLC profiling (see Chapters 10 and 12) can help choose an appropriate isolation protocol. The nature of the extract also provides clues that can be useful for choosing the right isolation protocol. For example, an EtOH or MeOH extract or the fractions from this extract contain polar compounds, and these polar compounds are better dealt with using reversed-phase HPLC.

Some physical properties of the extracts can be determined using a small portion of the crude extract in a series of small batch-wise experiments as outlined below.

(a) *Hydrophobicity or hydrophilicity*: An indication of the polarity of the extract as well as the compounds present in the extract can be obtained by drying an aliquot of the mixture and trying to redissolve it in various solvents covering the range of polarities, e.g., water, MeOH, acetonitrile (ACN), EtOAc, DCM, $CHCl_3$, petroleum ether, and *n*-hexane. Same information can be gathered by performing a range of solvent partitioning, usually between water and EtOAc, $CHCl_3$, DCM, or *n*-hexane, followed by assay to determine the distribution of compounds in solvent fractions.

(b) *Acid–base properties*: By partitioning in aqueous solvents at a range of pH values, typically 3, 7, and 10, it is possible to obtain information on the acid–base property of the compounds present in an extract or a fraction. It is necessary to adjust the aqueous solution or suspension with a drop or two of mineral acid or alkali (a buffer can also be used), followed by addition of organic solvent and solvent extraction. Organic and aqueous phases are assessed for the presence of certain compounds, usually, by TLC, or by analytical HPLC. This experiment may also provide information on the stability of compounds at various pH values.

(c) *Charge*: Information on charge properties of compounds can be obtained by testing the effect of adding various ion exchang-

ers to the mixture under batch conditions. This information is particularly of great importance when designing any isolation protocol that involves ion exchange chromatography (see Chapter 8).

(d) *Heat stability*: A typical heat stability test involves incubation of the sample at ~90°C for 10 min in a water bath followed by assay for unaffected compounds. It is particularly important for bioassay-guided isolation, where breakdown of active compound often results in the loss or reduction of biological activity. If the initial extraction of natural products is carried out at a high temperature, e.g., boiling, the test for heat stability becomes irrelevant.

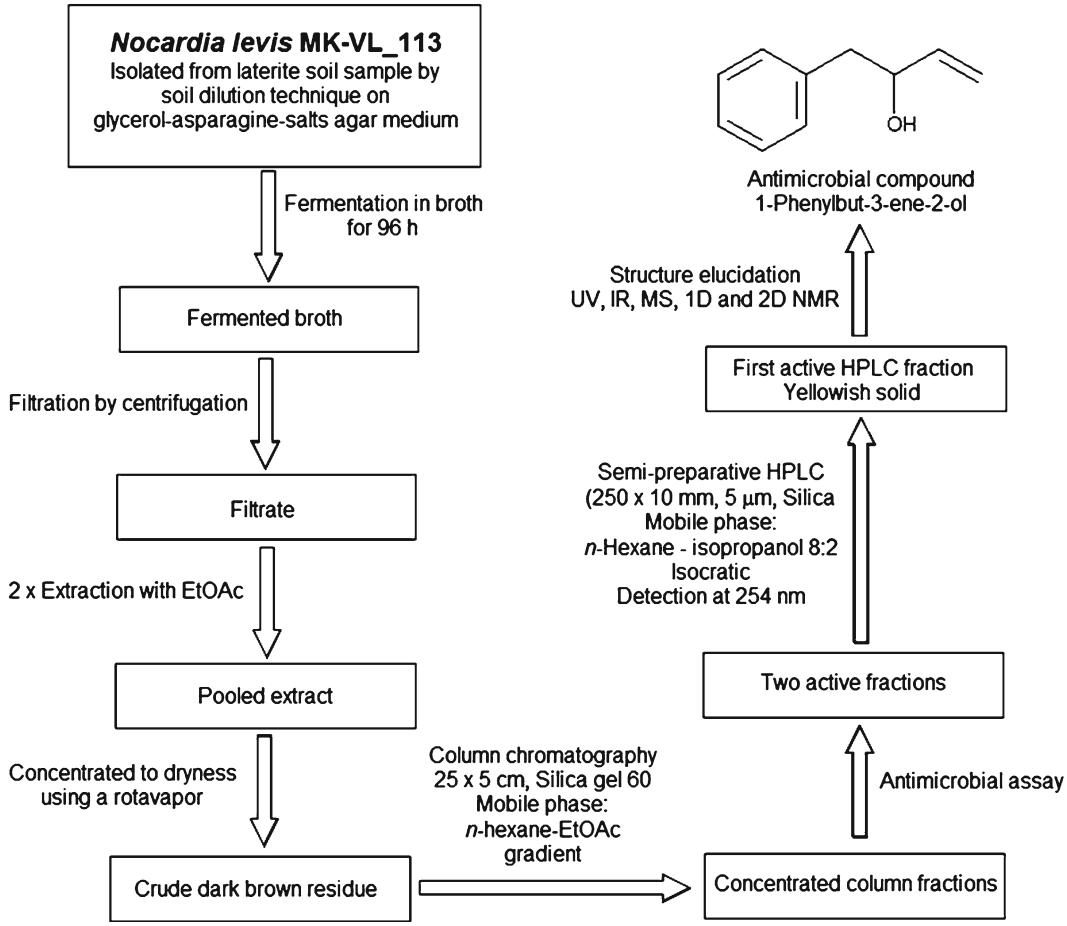
(e) *Size*: Dialysis tubing can be used to test if there are any macromolecules, e.g., proteins, present in the extract. Macromolecules are retained within the tubing allowing small (<2,000 amu) secondary metabolites to pass through the tubing. The necessity of using SEC in the isolation protocol can be ascertained in this way.

The chromatographic techniques used in the isolation of various types of natural products may be classified broadly into two categories, classical or older chromatographic techniques, and modern chromatographic techniques. The classical or older chromatographic techniques include FC, CC, SEC, TLC, PTLC, and ion-exchange chromatography, whereas chromatotron, DCC, high performance thin layer chromatography (HPTLC), multi-FC (e.g., Biotage®), HPLC, SPE, VLC, and a number of hyphenated techniques (e.g., HPLC–PDA, LC–MS, LC–NMR, LC–MS–NMR) may be considered to be modern chromatographic techniques. The details about most of the above techniques and their applications in isolation of natural products can be found in subsequent chapters of this book. Some specific examples of isolation protocols are summarized in Schemes 2 and 3 (12, 13) and a few more are outlined below (14–16).

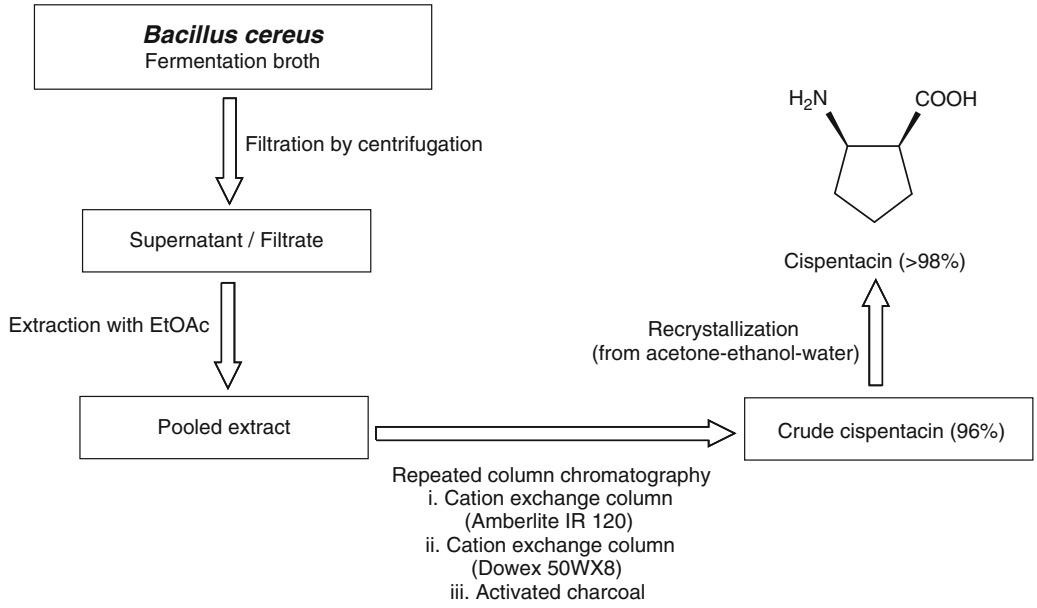
3.3.1. Isolation of Phytoecdysteroids from Limnanthes douglasii: A Bioassay-Guided Approach

A simple method for the isolation of two phytoecdysteroid glycosides, limnantheosides A and B, and two phytoecdysteroids, 20-hydroxyecdysone and ponasterone A (Fig. 1), using a combination of solvent extraction, SPE and preparative reversed-phase HPLC was reported by Sarker et al. (14).

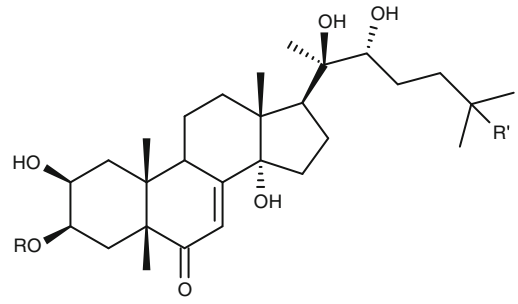
1. Grind the seeds (50 g) of *L. douglasii* and extract (4×24 h) with 4×200 mL of MeOH at 50°C with constant stirring using a magnetic stirrer. Pool the resulting extracts together.
2. Add sufficient amounts of water to make it a 70% aq. methanolic solution.
3. Perform defatting with *n*-hexane using solvent extraction (partitioning) technique.



Scheme 2. Isolation of the antimicrobial compound 1-phenylbut-3-ene-2-ol from *Nocardia levis* using a bioassay-guided approach (12).



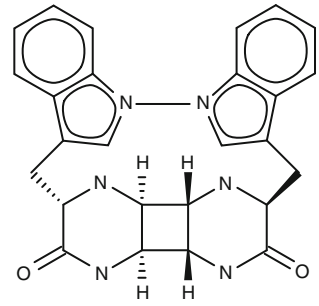
Scheme 3. Isolation of microbial natural product cispentacin from *Bacillus cereus* (13).



Compound	R	R'
Limnantheoside A	Xylosyl	OH
20-Hydroxyecdysone	H	OH
Limnantheoside B	Xylosyl	H
Ponasterone	H	H

Fig. 1. Phytoecdysteroids from *Limnanthes douglasii.*

4. Concentrate the aq. MeOH extract using a rotary evaporator.
5. Carry out SPE [on Sep-Pak Vac 35 cc (10 g) C_{18} cartridge (Waters)] of the concentrated extract (redissolved in 10% aq. MeOH) using MeOH–H_2O step gradient to obtain fractions.
6. Perform ecdysteroid bioassay/radioimmuno assay (RIA) to confirm the presence of ecdysteroids in the 60% MeOH–H_2O SPE fraction.
7. Carry out prep-HPLC analysis of the 60% MeOH–H_2O SPE fraction using a preparative reversed-phase C_8 column (Technoprep 10C8, 150 mm × 21.4 mm, 10 µm) and an isocratic elution with 55% MeOH–H_2O, 5 mL/min, monitored at 240 nm, to yield five fractions.
8. Perform ecdysteroid bioassay/RIA with the prep-HPLC fractions.
9. Subject the bioassay-positive fractions 2 (t_R 18–20 min) and 3 (t_R 33–36 min) to normal-phase (NP) semipreparative HPLC analyses (Apex II semiprep diol column, 150 mm × 10 mm, 5 µm, isocratic elution with 6% MeOH in DCM, 2 mL/min, detection at 240 nm) to obtain 20 hydroxyecdysone (purity >99%, t_R 13.1 min) and limnantheoside A (purity >99%, t_R 19.2 min) from fraction 2, and ponasterone A (purity >99%, t_R 5.2 min) and limnantheoside B (purity >99%, t_R 10.8 min) from fraction 3.



Schischkinin

Fig. 2. Schischkiniin from the seeds of *Centaurea schischkinii*.

3.3.2. Isolation of Schischkinin from Centaurea schischkinii

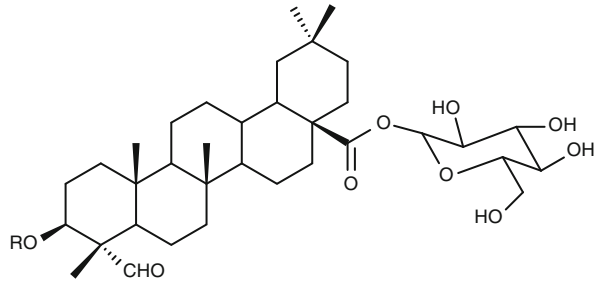
Reversed-phase HPLC analysis of the MeOH extract of the seeds of *Centaurea schischkinii* produced an unusual indole alkaloid schischkiniin (Fig. 2) (15). The protocol for this isolation is summarized below.

1. Grind the seeds of *C. schischkinii* (80 g), and perform the Soxhlet extraction, successively, with *n*-hexane, DCM, and MeOH (1 L each).
2. Concentrate the resulting extracts using a rotary evaporator.
3. Fractionate the MeOH extract by the SPE technique using a Sep-Pak C_{18} (10 g) cartridge eluting with a step gradient: 40, 60, 80, and 100% aq. MeOH (200 mL each).
4. Perform prep-HPLC analyses of the 40% SPE fraction on a Luna C_{18} preparative (10 μM, 250 mm × 21.2 mm) column eluting with a linear gradient, water:MeOH = 65:25–30:70 over 50 min followed by 70% aq. MeOH for 10 min (15 mL/min, monitor by photo-diode-array detector) to isolate schischkinin (t_R 8.1 min).

3.3.3. Isolation of Triterpene Saponins from Chenopodium quinoa

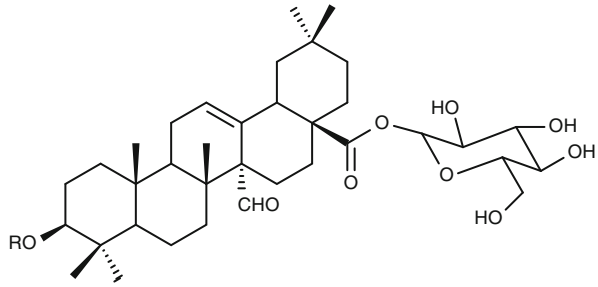
Four triterpene sapoinins, 3β-[(*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl)oxy]-23-oxo-olean-12-en-28-oic acid β-D-glucopyranoside, 3β-[(*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl)oxy]-27-oxo-olean-12-en-28-oic acid β-D-glucopyranoside, 3-*O*-α-L-arabinopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester, and 3-*O*-β-D-glucuronopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester were isolated from the *Chenopodium quinoa* (Fig. 3) (16). The isolation protocol is summarized below as follows:

1. Extract ground fruits (140 g) with MeOH by exhaustive maceration (3 × 2.5 L) and concentrate the extracts by a rotary evaporator.
2. Dissolve the methanolic extracts in water, and partition between EtOAc and *n*-BuOH, and dry the organic extracts completely.



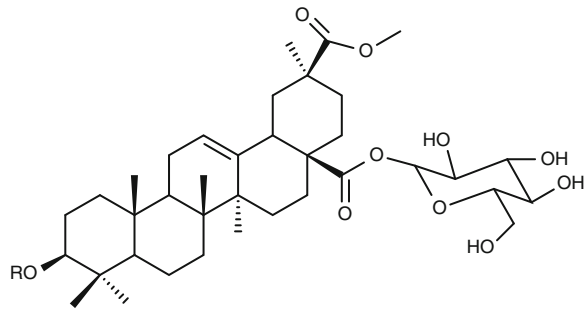
3*β*-[(*O*-*β*-D-glucopyranosyl-(1 →3)-α-L-arabinopyranosyl)oxy]-23-oxo-olean-12-en-28-oic acid β-D-glucopyranoside

R = -*β*-D-glucopyranosyl-(1 →3)-α-L-arabinopyranosyl



3β-[(*O*-β-D-glucopyranosyl-(1 →3)-α-L-arabinopyranosyl)oxy]-27-oxo-olean-12-en-28-oic acid β-D-glucopyranoside

R = -*β*-D-glucopyranosyl-(1 →3)-α-L-arabinopyranosyl



3-*O*-α-L-arabinopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester

R =-α-L-arabinopyranosyl

3-*O*-β-D-glucuronopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester

R =-β-D-glucuronopyranosyl

Fig. 3. Triterpene saponins from *Chenopodium quinoa*.

3. Redissolve the *n*-BuOH extract in *n*-BuOH saturated with water and perform a CC (column 400 mm × 35 mm) with 40–60 μm of silica gel, eluting with $CHCl_3$–MeOH–H_2O (7.5:2.3:0.2 4:5:1 *v*/*v*/*v*).
4. Collect 10 mL fractions and check by TLC on silica gel, developed with $CHCl_3$–EtOH–H_2O (4:2:0.4 *v*/*v*/*v*). Spray TLC plates with the Liebermann–Burchard reagent and heat to 120°C in an oven for 3 min.
5. Combine fractions showing similar profiles.
6. Perform reversed-phase chromatography (400 mm × 25 mm, 40–53 μm, Lichrosoher 100 C_{18} column, eluting with aq. ACN) on column fractions. Fractions eluted with 20% aq. ACN will give 3β-[(*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl)oxy]-23-oxo-olean-12-en-28-oic acid β-D-glucopyranoside and 3β-[(*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl)oxy]-27-oxo-olean-12-en-28-oic acid β-D-glucopyranoside, while 3-*O*-α-L-arabinopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester and 3-*O*-β-D-glucuronopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester will be found in the fraction eluted with 25% aq. ACN.

3.4. Quantification of Yield

The yield of compounds at the end of the isolation and purification process is important in natural products isolation. An estimate of the recovery at the isolation stage can be obtained by various analytical techniques that may sometime involve the use of a standard. In bioassay-guided isolation, the compound is monitored by bioassay at each stage, and a quantitative assessment of bioactivity of the compound is usually carried out by serial dilution method (see Note 5). Quantitative bioactivity assessment presents a clear idea about the recovery of the active compound(s), and also indicates whether the activity is due to a single or multiple components. During the isolation process, if the activity is lost or reduced to a significant level, the possible reason(s) could be one or more of the following:

(a) The active compound is retained in the column.

(b) The active compound is unstable in the conditions used in the isolation process.

(c) The extract solution may not have been prepared in solvent that is compatible with the mobile phase so that a large proportion of the active components precipitated out when loading on to the column.

(d) Most of the active component(s) is spread across a wide range of fractions causing undetectable amounts of component(s) present in the fractions.

(e) The activity of the extract is probably due to the presence of synergy among a number of compounds, which when separated, are not active individually.

3.5. "Poor Yield" Issue

"Poor yield" or "poor recovery" is one of the major problems in natural products isolation, especially when the active compound is present in extremely low concentration in a natural product extract. For example, only 30 g of vincristine was isolated from 15 tons of dried leaves of *Vinca rosea* (or *C. roseus*) (17). Similarly, in order to obtain 1,900 g of Taxol®, it had required the felling of 6,000 trees to acquire 27,300 kg of the bark from the extremely slow growing tree, *T. brevifolia*. To deal with this "poor-yield" issue, one may adopt one of the following approaches:

1. Find a better source for the supply of the target compound. The source may be a different species or a cultivar of the same genus, a different plant part or cultivation conditions.
2. Use genetic manipulation of the source.
3. Use semisynthesis of the target compound from a more abundant precursor.
4. Perform total synthesis of the target compound.
5. Utilize tissue or cell culture production.

3.6. Structure Elucidation of Isolated Compounds

Isolated natural compounds are identified or characterized by conclusive structure elucidation techniques. However, structure elucidation of natural products is generally a time-consuming process, and sometimes can be the "bottleneck" in natural products research. It is probably not much of a problem for well-known natural products, but it can certainly be challenging at times, if the compounds are new entity. There are many useful spectroscopic methods that provide valuable information about chemical structures, but the interpretation of these spectra requires specialist spectroscopic knowledge, structure elucidation skills, sound understanding of natural products chemistry, and above all, a great deal of patience. With the remarkable advances in the area of artificial intelligence and computing, nowadays, there are several useful automated structure elucidation programs available which could be extremely helpful (18–20). However, none of the programs may not necessarily replace the years of "hands on" experience of a natural products chemist!

If the target compound is known, the structure can be determined often easily by comparing its preliminary spectroscopic data with literature data or direct chromatographic comparison with the standard sample. However, if the target compound is an unknown and complex natural product, e.g., Taxol®, a combination of physical, chemical, and spectroscopic data analyses is required for structure elucidation. Also, information on the chemistry of the genus or the

family of plant or microbe under investigation could sometimes provide important clues regarding the possible chemical class of the unknown compound. The following spectroscopic techniques are routinely employed for structure determination of natural products.

1. *Ultraviolet–visible spectroscopy (UV–vis)*: Provides information on chromophores present in the molecule. Some natural products, e.g., coumarins, flavonoids, isoquinoline alkaloids, or phytoecdysteroids, can be primarily characterized (chemical class) from characteristic absorption peaks.
2. *Infrared spectroscopy (IR)*: Different functional groups, e.g., –C=O, –OH, $–NH_2$, or aromaticity present in a molecule can be determined.
3. *Mass spectrometry (MS)*: Gives information about the molecular mass, molecular formula, and fragmentation pattern. Most commonly used techniques are, electron impact mass spectrometry (EIMS), chemical ionization mass spectrometry (CIMS), electrospray ionization mass spectrometry (ESIMS), fast atom bombardment mass spectrometry (FABMS), or matrix-assisted laser desorption ionization (MALDI).
4. *NMR*: Provides information on the number and types of protons and carbons (and other elements, like nitrogen and fluorine) present in the molecule, and the relationships among these atoms (21, 22). The NMR experiments that are used routinely to elucidate the structures of natural products can be classified into two major categories.
   (a) One-dimensional NMR techniques: ^{1}H NMR, ^{13}C NMR, ^{13}C DEPT, ^{13}C PENDANT, ^{13}C *J*-mod., and nOe-diff.
   (b) Two-dimensional NMR: ^{1}H–^{1}H COSY, ^{1}H–^{1}H DQF-COSY, ^{1}H–^{1}H COSY-lr, ^{1}H–^{1}H NOESY, ^{1}H–^{1}H ROESY, ^{1}H–^{1}H TOCSY (or HOHAHA), ^{1}H–^{13}C HMBC, ^{1}H–^{13}C HMQC, ^{1}H–^{13}C HSQC, and HSQC-TOCSY.

In addition, X-ray crystallography provides information on the crystal structure of the molecule, and polarimetry offers information on the optical activity of chiral compounds. These two techniques are particularly important for molecules with chiral centers and optical isomerism.

3.7. Assays

Chemical, biological, or physical assays are often associated with natural products isolation. Assays are particularly important for assay-guided isolation of natural products. Nowadays, natural products isolation is predominantly about isolating target compound utilizing assay-guided approach rather than thorough isolation. The target compounds are of certain chemical classes, have certain physical properties or may possess certain biological activities. Therefore, appropriate assays are required for successful isolation

of the target compounds. The following basic points should be borne in mind when incorporating assays in any natural products isolation protocol and carrying out assays with natural products (23).

1. Samples dissolved or suspended in a solvent different from the original extraction solvent must be filtered or centrifuged, to get rid of any insoluble matter.
2. Acidified or basified samples should be readjusted to their original pH to prevent them from interfering with the assay.
3. Positive and negative controls must be incorporated in any assay.
4. Ideally, the assay should be at least semiquantitative, and/or samples should be assayed at a series of dilutions in order to determine where the majority of the target compounds reside.
5. The assay must be sensitive enough to detect active components in low concentration.

Physical assays may involve the comparison of various chromatographic and spectroscopic behaviors of the target compound with a known standard. For example, the retention time or retention indices of the target compound can be compared with that of the standard. Chemical assays involve various chemical tests for identifying the chemical nature or chemical class of any compound, e.g., $FeCl_3$ can be used to detect phenolics, the Dragendorff's reagent for alkaloids (see Chapter 6) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay to assess the free radical scavengers (24, 25).

Bioassays can be defined as the use of a biological system to detect any activities of test samples, e.g., antibacterial, antifungal, anticancer, anti-HIV, and antidiabetic activities. The test sample can be a crude extract, a chromatographic fraction, a mixture or a pure compound. Bioassays may involve the use of in vivo systems (clinical trials, whole animal experiments), ex vivo systems (isolated tissues and organs), or in vitro system (e.g., cultured cells). In vivo studies are more relevant to clinical conditions and also can provide toxicity data at the same time. However, there are some disadvantages of this assay, which include high cost, need for large amount of test compounds/fractions, complex design, animal or patient requirements, and difficulty in mode of action determination. Moreover, in most in vivo bioassays, ethical approvals are needed from appropriate authorities.

In vitro bioassays are faster (ideal for HTS), and small amounts of test compounds are needed. However, this type of assays but may not be relevant to clinical conditions. Many of the in vitro bioassays available today are robust, specific, and ultrasensitive; even the bioactivity due to as low as picogram amounts of test compounds can easily be detected by some assays. Many of them can be performed in full or semiautomation (e.g., using 96- or 384-well plates). There are a number of biological assays available to assess various activities, e.g., *Drosophila melanogaster* B_{II} cell

line assay for the assessment of compounds with ecdysetorid (see Note 6) agonist or antagonist activity (26), antibacterial serial dilution assay using resazurin as an indicator of cell growth (11, 27). Most of the modern bioassays are microplate based and require small amount of extract, fraction, or compound for the assessment of activity. While it is not the intention of this chapter to discuss various assays available to date, the protocols of three typical assays used in natural products screening, the DPPH assay, antibacterial serial dilution assay using resazurin as an indicator of cell growth, and heme biocrystallization or polymerization assay, are presented here as examples. Details on various types of bioassays used in the screening of natural products are available in the literature (28).

3.7.1. The DPPH Assay for Free Radical Scavengers (24, 25)

DPPH, molecular formula $C_{18}H_{12}N_5O_6$, is used in this assay to assess the free radical scavenging (antioxidant) property of natural products. Quercetin, a well-known natural antioxidant, or Trolox® is generally used as a positive control.

1. Dissolve DPPH (8 mg) in MeOH (100 mL) to obtain a concentration of 80 μg/mL.
2. Prepare the solution (1 mg/mL) of the positive standard in MeOH.
3. Prepare the stock solution of test samples in MeOH; 10 mg/mL for crude extracts and fractions, and 1 mg/mL for purified compounds (see Note 7).
4. First carry out qualitative assay and then perform quantitative assay only with the samples that show positive result in the quantitative assay.
5. For qualitative assay, apply test extracts, fractions, or compounds on a silica gel TLC plate and spray with the DPPH solution using an atomizer. Leave the TLC plate for 30 min to develop. White spots against a pink background indicate the presence of free radical scavengers (see Note 8).
6. For quantitative assay, carry out serial dilution (tenfold) of the test solutions as well as the positive control solution to obtain concentrations of 1.0, 0.1, 0.001, 0.0001, and 0.00001 mg/mL. Use a vortex machine to mix diluted solutions (1.00 mL each) with DPPH (1.00 mL). For the blank, mix 1 mL of MeOH and 1 mL of DPPH solution. Allow the mixtures to stand for 30 min for any reaction to occur. Record the absorbance of these solutions at 517 nm using a UV–vis or visible bench-top spectrophotometer. Perform the experiment in triplicate and note the average absorbance for each concentration. Finally, calculate the percentage inhibition of DPPH absorption by each dilution using the following equation.

   $\%\ \text{Inhibition} = [(B - A) / B] \times 100$, where B and A are the absorbance of the blank and the test solution at 517 nm.

3.7.2. Microtiter Plate-Based Antibacterial Assay Incorporating Resazurin as an Indicator of Cell Growth (11)

1. Use either isosensitest medium or Mueller Hinton medium.
2. To ensure that a uniform number of bacteria are always used, prepare a set of graphs of killing/viability curves for each strain of bacterial species. Use a final bacterial concentration of 5×10^5 cfu/mL for this assay.
3. Prepare resazurin (the indicator) solution by dissolving a 270 mg resazurin tablet in 40 mL of sterile distilled water. Use a vortex mixer to ensure that it is a well-dissolved and homogenous solution.
4. Prepare bacterial culture in the following way.
   (a) Using aseptic techniques, transfer a single colony into a 100 mL bottle of isosensitest broth, cap it, and place it in incubator overnight at 35°C. After 12–18 h of incubation, using aseptic preparation and the aid of a centrifuge, a clean sample of bacteria is prepared.
   (b) Spin down the broth using a centrifuge set at 4,000 rpm for 5 min with appropriate aseptic precautions. Discard the supernatant into an appropriately labeled contaminated waste beaker.
   (c) Resuspend the pellet using 20 mL of sterile normal saline and centrifuge again at 4,000 rpm for 5 min. Repeat this step until the supernatant is clear. Suspend the pellet again in 20 mL of sterile normal saline, and label as Bs.
   (d) Record the optical density of the Bs at 500 nm, and perform serial dilutions with appropriate aseptic techniques until the optical density is in the range of 0.5–1.0. The actual number of colony-forming units can be calculated from the viability graph.
   (e) Calculate the dilution factor needed and carry out dilution to obtain a concentration of 5×10^6 cfu/mL.
5. Prepare the microtiter-plates under aseptic conditions and perform the assay using the following easy steps.
   (a) Label a sterile 96-well plate vertically (Fig. 4).
   (b) Pipette a volume of 100 µL of test material in 10% (*v*/*v*) DMSO or sterile water (usually a stock concentration of 1 mg/mL for purified compounds and 10 mg/mL for crude extracts) into the first row of the plate.
   (c) To all other wells, add 50 µL of nutrient broth or normal saline.
   (d) Perform serial dilutions preferably using a multichannel pipette such that each well has 50 µL of the test material in serially descending concentrations. Discard tips after use.
   (e) To each well, add 10 µL of resazurin indicator solution.
   (f) Using a pipette, add 30 µL of 3.3× strength isosensitest broth to each well to ensure that the final volume is single strength of the nutrient broth.

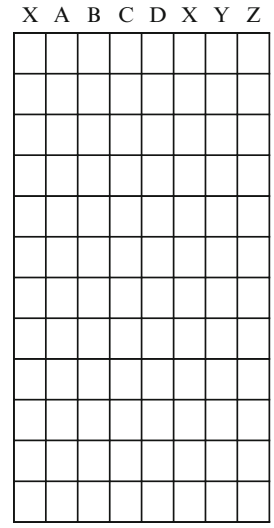


Fig. 4. Microtiter plate preparation layout [*X* = sterility control (test compound in serial dilution + broth + saline + indicator), no bacteria; *Y* = control without drug (bacteria + broth + indicator); *Z* = positive control (ciprofloxacin in serial dilution + broth + indicator + bacteria); *A–D* = test compound/extract (in serial dilution in wells 1–12 + broth + indicator + bacteria)].

(g) Finally, add 10 μL of bacterial suspension (5×10^6 cfu/mL) to each well to achieve a concentration of 5×10^5 cfu/mL.

(h) Wrap each plate loosely with cling film to ensure that bacteria did not become dehydrated. Each plate should have a set of controls: a column with a broad-spectrum antibiotic as positive control (usually ciprofloxacin in serial dilution), a column with all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution adding 10 μL of nutrient broth instead.

(i) Prepare the plates in triplicate and place in an incubator set at 37°C for 18–24 h.

(j) Assess the color change visually. Any color changes from purple to pink or colorless should be recorded as positive. The lowest concentration at which color change occurs is taken as the MIC value.

(k) Calculate the average of three values and this is the MIC for the test material and bacterial strain.

3.7.3. Heme Biocrystallization or Polymerization Assay (29, 30)

The potential antimalarial activity of natural products can be evaluated by the heme biocrystallization or polymerization assay (29). The protocol can be outlined as follows:

1. Prepare test sample at concentrations of 0.01–10 mg/mL in 10% DMSO.

2. Incubate test sample (100 μL) with 100 μL of 3 mM hematin (freshly dissolved in 0.1 M NaOH), 10 mM oleic acid, 10 μL of 1 M HCl.
3. After adding the test samples at varying concentrations, adjust the reaction volume to 1,000 μL using 500 mM sodium acetate buffer of pH 5.
4. Use chloroquine diphosphate as a positive control with the negative control containing buffer without test compounds.
5. Incubate the samples for 4 h with gradual shaking/inverting of each tube.
6. After incubation, centrifuge the samples (14,000 rpm, 10 min, at 21°C) and wash the hemozoin pellets repeatedly with 2% (*w*/*v*) SDS in 0.1 M sodium bicarbonate, pH 9.0, with sonication (30 min, at 21°C), until the supernatant is clear (usually 3–5 times).
7. After the final wash, remove the supernatant and resuspend the pellets in NaOH (0.1 M, 1 mL,) and incubate for an additional hour at room temperature.
8. Thereafter, vortex the samples and determine the hemozoin content by measuring the absorbance at 400 nm using a 1 cm quartz cuvette. The concentration of drug/compound/extract required to produce 50% inhibition of polymerization (IC_{50}) can be determined graphically.

4. Notes

1. The word "Ayurveda" means "Knowledge of long life," and the Ayurvedic medicine is a system of Indian traditional medicine.
2. "Drug-like properties" refers to the fact that the molecules are absorbed and metabolized like conventional drugs in human body.
3. The conclusive structure elucidation of an unknown natural product using high field modern 1D and 2D NMR techniques requires the compound to be pure >90%. For a compound of known structure, the structure can be deduced from a less pure compound. In X-ray crystallographic studies, materials are required in an extremely pure state, >99.9% pure. For bioassays, it is also important to know the degree of purity of the test compound. The most reliable assay result can be obtained with a compound with ~100% purity because it excludes any possibilities of having the activity due to minor impurities.

4. If the extraction is designed only to produce enough material for confirmation or denial of a proposed structure of a previously isolated compounds, it may require less material or even partially pure material, because in many cases this does not require mapping out a complete structure from scratch but perhaps simply comparison with a standard of known structure.
5. Approximate quantification can be performed by assaying a set of serial dilutions of each fraction at each stage of the separation process. To detect the peaks of activity, it is often necessary to assay the fractions at a range of dilutions, which indicate the relative amounts of activity (proportional to the amount of compound present) in each fraction. Thus, the fraction(s) containing the bulk of the active compounds can be identified, and an approximate estimation of the total amount of activity recovered, relative to starting material, can be obtained.
6. Ecdsyteroids are insect molting hormones, and they are also found in various plant species.
7. The solution of fully characterized pure natural compounds as well as positive control may also be prepared at micromolar concentrations instead of milligram per milliliter.
8. TLC plates with crude test extracts or fractions may be first developed using a suitable mobile phase to separate any compounds, dried and then finally sprayed with the DPPH solution to locate free radical scavengers on the plate.

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